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Resurrecting Van Leeuwenhoek's rotifers: a reappraisal of the role of disaccharides in anhydrobiosis

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In 1702, Van Leeuwenhoek was the first to describe the phenomenon of anhydrobiosis in a species of bdelloid rotifer, *Philodina roseola*. It is the purpose of this review to examine what has been learned since then about the extreme desiccation tolerance in rotifers and how this compares with our understanding of anhydrobiosis in other organisms. Remarkably, much of what is known today about the requirements for successful anhydrobiosis, and the degree of biostability conferred by the dry state, was already determined in principle by the time of Spallanzani in the late 18th century. Most modern research on anhydrobiosis has emphasized the importance of the non-reducing disaccharides trehalose and sucrose, one or other sugar being present at high concentrations during desiccation of anhydrobiotic nematodes, brine shrimp cysts, bakers' yeast, resurrection plants and plant seeds. These sugars are proposed to act as water replacement molecules, and as thermodynamic and kinetic stabilizers of biomolecules and membranes. In apparent contradiction of the prevailing models, recent experiments from our laboratory show that bdelloid rotifers undergo anhydrobiosis without producing trehalose or any analogous molecule. This has prompted us to critically re-examine the association of disaccharides with anhydrobiosis in the literature. Surprisingly, current hypotheses are based almost entirely on *in vitro* data: there is very limited information which is more than simply correlative in the literature on living systems. In many species, disaccharide accumulation occurs at approximately the same time as desiccation tolerance is acquired. However, several studies indicate that these sugars are not sufficient for anhydrobiosis; furthermore, there is no conclusive evidence, through mutagenesis or functional knockout experiments, for example, that sugars are necessary for anhydrobiosis. Indeed, some plant seeds and micro-organisms, like the rotifer, exhibit excellent desiccation tolerance in the absence of high intracellular sugar concentrations. Accordingly, it seems appropriate to call for a re-evaluation of our understanding of anhydrobiosis and to embark on new experimental programmes to determine the key molecular mechanisms involved.

Keywords: desiccation; desiccation tolerance; trehalose; sucrose

1. INTRODUCTION

Three hundred years ago, in a letter to Hendrik van Bleyswijk dated 9 February 1702, the pioneering microscopist Antony van Leeuwenhoek (figure 1) was the first to describe a remarkable phenomenon. He discovered that when dry and apparently lifeless dust from a roof gutter was rehydrated with clean water in a glass tube, many small 'animalcules' became active within an hour, variously 'adhering to the glass, some creeping along it, and some swimming about.' This result, observed with his characteristic single-lens microscopes, was routinely reproducible, even with dust kept dry for many months. Van Leeuwenhoek was astonished: 'I confess I never thought that there could be any living creature in a substance so dried as this was' (p. 209) (Van Leeuwenhoek 1702, translated by Hoole 1807; Van Rijnberk & Palm 1999). His astonishment is understandable, as water is recognized as the 'matrix of life' (Szent-Györgyi 1979; Franks 2000). Without water, there can be no life, and yet Van Leeuwenhoek is describing creatures which apparently survive desiccation.

The drawings included with Van Leeuwenhoek's letter (figure 2*a*) show that his animalcule was what Baker (1744, 1753) later called a 'wheel animal', in reference to the coronal cilia whose metachronal beat gives the impression of two rotating wheels on the animal's head. The term in current use for these creatures was introduced first in Italian by Felice Fontana ('rotifero'; Fontana 1767) and derives from the Latin for 'wheel bearer'. A photograph of the rotifer *Philodina roseola* (figure 2*b*) illustrates the resemblance to Van Leeuwenhoek's specimen. Indeed, given that *P. roseola* often appears orange or red (hence roseola, 'rose-coloured') owing to its diet of the astaxanthin-containing phytoflagellate of the same colour, *Haematococcus pluvialis*, and that it inhabits temporary aqueous environments (*Philodina* means 'lover of pools'), it is highly likely that Van Leeuwenhoek's red, gutter-dwelling animalcule was of the same species (Ford 1982; Koste & Hollowday 1993; Van Rijnberk & Palm 1999).

Rotifers occupy a separate phylum, Rotifera, comprising three classes, with *P. roseola* belonging to the Bdelloidea. Bdelloid ('leech-like') rotifers are swimming or creeping

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Figure 1. Antony van Leeuwenhoek at the age of 54, from a mezzotint reproduction of an oil painting by Johannes Verkolje 1686 (The Royal Society collection). Reproduced with permission.

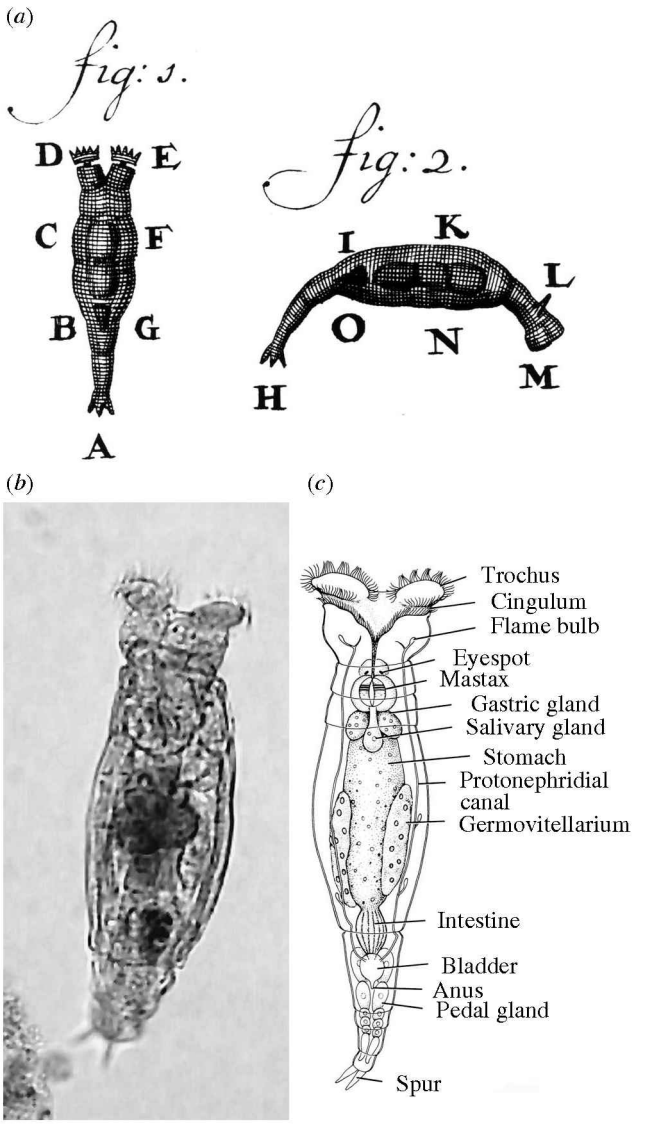

Figure 2. The bdelloid rotifer *Philodina roseola*. (*a*) Drawings of the rotifers that Van Leeuwenhoek (1702) observed. (*b*) A photograph of *P. roseola* obtained from a bird bath. (*c*) The structure of a bdelloid rotifer, reproduced from Pierce *et al.* (1987), with permission.

unsegmented pseudocoelomates, ranging in size from 100 to 2000 μm; the body (figure 2*c*) is divided into three regions: the foot with toes, the trunk, and the head bearing the characteristic cilia whose action induces a vortex in water, drawing small food particles towards the mouth. Bdelloids inhabit freshwater ponds, lakes, temporary pools, brackish waters, sewage and the interstices of soil, mosses and lichens (Ricci 1987). Some of these habitats dry out frequently, and the ability of most bdelloid species to withstand desiccation and the dry state (Ricci 1998) must represent a clear selective advantage. This ability—survival of extreme desiccation and entry into a state of suspended animation, pending recovery by rehydration—is known as anhydrobiosis, a term derived from the Greek for 'life without water'.

Van Leeuwenhoek had described rotifers in earlier correspondence with The Royal Society, most clearly in his letter of 17 October 1687 (Van Rijnberk & Palm 1999), and had sent specimen packages with some letters, which were rediscovered by Ford (1981). When Ford rehydrated these specimens, some contained animals identifiable as rotifers, even allowing a proposed species designation, although none had apparently survived its three centuries of dryness (Ford 1982, 1991). Nevertheless, viability can be maintained for extended periods in the dry state, the current record for (well-documented) survival of a dried animal being of the order of decades for nematodes and brine shrimp cysts (Fielding 1951; Clegg 1967; Jönsson & Bertolani 2001). The longest authenticated period survived by a rotifer after desiccation appears to be 9 years (Guidetti & Jönsson 2002), although there is potential for a much longer phase of suspended animation: 1000-year-old lotus seeds have germinated successfully (Shen *et al.* 1995) and there are regular claims of the recovery of ancient bacteria up to 250 million years old (e.g. Vreeland 2000), although these claims are, equally regularly, debated (e.g. Nickle *et al.* 2002).

In the past, the tolerance of extreme dehydration and the dry state that anhydrobiosis represents has challenged some fundamental biological precepts, particularly regarding the necessity of water for life and the requirement for metabolic continuity, and has contributed to the debate on spontaneous generation (Keilin 1959). Modern research places emphasis on unravelling the molecular and cellular mechanisms underlying anhydrobiosis, as we are still a long way from a full understanding of these processes. This review will summarize what is known about anhydrobiosis in rotifers, with reference to other species, and include some surprising recent results which may necessitate modification of prevailing models.

2. ANHYDROBIOSIS

(a) *Latent life*

The term anhydrobiosis was introduced by Giard (1894) and refers specifically to the highly stable state of suspended animation that an organism enters by desiccation. Strictly, therefore, it is distinct from 'desiccation tolerance', which refers to the ability of a cell or organism to withstand removal of water to a greater or lesser extent, although the terms are often used interchangeably. Anhydrobiosis is a particular form of cryptobiosis—meaning latent life or suspended animation—as defined by Keilin (1959). Other forms of cryptobiosis such as cryobiosis, anoxybiosis and osmobiosis are induced by cooling, lack of oxygen or high osmolarity in the environment, respectively.

Clegg (2001) has argued that '[a]nhydrobiosis tells us something fundamental about the basic nature of living systems' (p. 615). In the dry state, there is little or no evidence of metabolic activity (Keilin 1959; Clegg 1986). Indeed, when a cell has a water content of less than 0.1 g g dry mass^{-1} (*ca.* 9% w/w), an amount typically remaining in dried but viable anhydrobiotic organisms, it has been calculated that there is insufficient water to fully hydrate intracellular proteins, and that therefore metabolism is impossible (Clegg 1978, 1986; Potts 1994). Normally, of course, an organism without metabolism is considered dead, and irreversibly so. However, in cryptobiotic, and specifically here in anhydrobiotic, organisms, the cessation of metabolism is fully reversible, and a kind of resurrection is routinely performed. This has led Clegg (2001) to conclude that 'there are three states of biological organization: alive; dead; and cryptobiotic' (p. 615).

Given this, we might briefly reflect on nomenclature: Keilin (1959) discussed the diverse expressions in use in the early literature and pointed out the problems associated with a number of them ('abiosis', 'anabiosis', etc.). However, if dried anhydrobiotic organisms are neither dead nor alive, but in suspended animation, his adoption of Giard's term (i.e. 'anhydrobiosis') is also unsatisfactory, as is the extension of this construction to other cryptobiotic states ('cryobiosis', etc). While 'cryptobiosis' is fine, because it tells us that life processes are in a latent condition, 'anhydrobiosis' is not, because it does not convey the idea of latency. We are not describing life without water, which implies some biology continuing in the dry state, but a metabolic stasis (or 'biostasis') where the organism has shut down completely. 'Anhydrous cryptobiosis' would be more accurate, but too cumbersome. Something like 'anhydrobiostasis', or the shorter 'anhydrostasis', might be more suitable. This argument can be taken a step further, because the prefix 'anhydro-' means, literally, 'without water', although dried anhydrobiotes always retain some water and are therefore never likely to be truly anhydrous outside the laboratory. 'Dry', however, is a relative term and could be used correctly in this context. The Greek prefix, 'xero-', would then lead to the construction 'xerobiostasis' or 'xerostasis'. Unfortunately, as 'anhydrobiosis' is firmly established in the literature, it is unlikely that it can now be replaced.

Anhydrobiosis is widespread in nature and in everyday experience, an example being the dried granules of bakers' yeast (*Saccharomyces cerevisiae*) obtainable from any supermarket (Schebor *et al.* 2000). Other anhydrobiotic micro-organisms include cyanobacteria (Potts 1999) and the radiotolerant *Deinococcus radiodurans* (Mattimore & Battista 1996). However, Potts (1994) has, with justification, rejected the acceptance of bacterial spores and akinetes into the anhydrobiotic family on the grounds that they contain too much water, and certainly values for the residual water content in the range 0.21–0.58 g H_2O g dry weight^{-1} could allow some continuation of biological processes (Clegg 1978; Algie & Wyatt 1984). In the animal kingdom, members of three invertebrate phyla—tardigrades (Wright 2001), nematodes (Perry 1999) and rotifers—are capable of anhydrobiosis at all developmental stages, including the adult form. Other invertebrates, including crustaceans such as *Artemia* spp., have anhydrobiotic embryonic cysts (Clegg 2001). The desiccated larvae of the fly *Polypedium vanderplanki* (Diptera) contain some 8% residual water and can be regarded as anhydrobiotic (Hinton 1960). Among plants, some algae are capable of anhydrobiosis (e.g. the encysted form of phytoflagellate *H. pluvialis*, a food source for Van Leeuwenhoek's rotifer, *P. roseola*), as are lichens and bryophytes, such as the star moss *Tortula ruralis*. The form of anhydrobiosis found in these primitive plants seems to comprise constitutively expressed cell protection mechanisms, together with inducible repair systems which are activated on rehydration (Oliver 1997). Most higher plants have lost vegetative desiccation tolerance, but some, for example the 'resurrection' plants *Selaginella lepidophylla* and *Craterostigma plantagineum*, reacquired it later in evolution in a modified form, as entry into anhydrobiosis requires metabolic adjustment during a slow dehydration phase (Ingram & Bartels 1996; Oliver & Bewley 1997; Oliver *et al.* 2000), implicating inducible protection mechanisms. These examples are anhydrobiotic in the vegetative state, but the seeds and, to a lesser extent, pollen of many flowering plants are also well known to survive long periods in the dry state, as seen in the example of the lotus seeds (above). It might be argued that anhydrobiosis in a whole plant is more impressive than in its seeds or pollen, as the latter are specialized forms whose development is intrinsically linked to the desiccation process. However, it is likely that anhydrobiosis in seeds and other propagules is a later evolutionary adaptation and that this is one example of multiple reacquisitions of desiccation tolerance throughout the evolution of the plant kingdom (Oliver & Bewley 1997; Oliver *et al.* 2000). The ability of members of all biological kingdoms to undergo anhydrobiosis could suggest that this is a property developed by ancient cell types and it may have been essential for efficient colonization of land masses.

(b) *Defining anhydrobiosis*

The question of which organisms are categorized as anhydrobiotic is not straightforward, at least for single-celled species, and relates to the degree of survival of the dehydration/rehydration cycle. In this regard, we define viability as the maintenance of reproductive capacity after desiccation and rehydration. Micro-organisms exhibit varying survival rates under different conditions (Potts 1994), but it is not clear where 'true' anhydrobiosis begins. For example, 60–100% of *D. radiodurans* (Mattimore & Battista 1996; Battista *et al.* 2001) and 0.5–

10% of cyanobacterium *Chroococcidiopsis* sp. (Grilli Caiola *et al.* 1993) populations survive desiccation, but both of these micro-organisms are recognized anhydrobiotes. By contrast, the enterobacterium *Escherichia coli* is not generally considered anhydrobiotic, with typical survival levels immediately after drying of 2–8% (e.g. Louis *et al.* 1994; Leslie *et al.* 1995). However, mucoid variants of *E. coli* can show 10-fold improvement in recovery rates (Ophir & Gutnick 1994), apparently better than *Chroococcidiopsis*. With suitable preconditioning of bacteria and adjustment of drying protocols, 50–80% viability of non-mucoid *E. coli* can be maintained for at least six weeks at above ambient temperatures (García de Castro *et al.* 2000*a*; Tunnacliffe *et al.* 2001). Is *E. coli* therefore capable of anhydrobiosis? Other factors are important, of course, such as the ability to survive in a 'real' environment, outside the laboratory; the degree of desiccation; and the length of time spent in the dry state (the 'storage' period). Nevertheless, the definition of anhydrobiosis in micro-organisms is nebulous and, although some species perform better than others, the suspicion is that interspecies differences are quantitative, rather than qualitative. This may ultimately frustrate the identification of specific adaptations to water stress in micro-organisms.

The degree of survival required for many unicellular organisms, where only a single individual survivor among billions desiccated might be sufficient to regenerate the population, is low compared with individual metazoans, where all or most cells must recover. The model organism *Caenorhabditis elegans*, a non-anhydrobiotic nematode, can remain viable even when a significant percentage of its 1000 or so somatic cells have been ablated or caused to apoptose or otherwise damaged. Therefore, if the same is true for the anhydrobiotic nematode *Aphelenchus avenae*, for example, a degree of cell death might be tolerated during entry into, and recovery from, anhydrobiosis. However, further rounds of anhydrobiosis would inflict increasing levels of cell damage and ultimately be fatal, because there is no, or little, capacity for tissue regeneration or cell division in mature nematodes (Wright 1991) as, indeed, is the case for post-embryonic rotifers (Clément & Wurdak 1991). Because it is known that, with suitable recovery periods, an anhydrobiotic animal can repeatedly survive desiccation and rehydration (Spallanzani 1776; Perry 1977; Schramm & Becker 1987), it is likely that essentially all somatic and germ cells are anhydrobiotic. The fact that most rotifer tissues are syncitial does not significantly alter this argument. The situation is different in plants, which can tolerate extensive cell damage and where differentiation continues post-embryonically from meristem tissue. In the case of the resurrection plants, however, significant cell death does not seem to occur during dehydration and rehydration (Gaff 1971). In multicellular animals and plants, therefore, it is likely that a large majority of, and possibly all, cells within the organism must be capable of, and survive, anhydrobiosis.

(c) *The biostability of anhydrobiosis*

In itself, the ability to survive without (much) water is noteworthy in itself, but, once in the dry state, anhydrobiotic organisms become extremely resistant to a number of stresses besides desiccation. Dried rotifers have remained viable after cooling to within a degree of absolute zero (Becquerel 1950) and heating to 151 °C (Rahm 1923); dried tardigrades exhibit similar thermotolerance and, in addition, have survived exposure to 6000 atmospheres of hydrostatic pressure (Seki & Toyoshima 1998) and to ionizing radiation (Rahm 1923, 1937; May *et al.* 1964). To a large extent, this extreme biostability is due to the absence of water, as most biochemistry—including degradative processes—proceeds through aqueous reactions. Otherwise thermolabile proteins which have been protected from damage during desiccation become extremely resistant to thermal stress in the dry state (Colaço *et al.* 1992). However, dried or partially dried biologicals are still susceptible to some chemistry such as the Maillard reaction (Maillard 1912; Lea *et al.* 1950) and the effects of ultraviolet radiation (e.g. Rahm 1923; Mancinelli & Klovstad 2000).

The remarkable characteristics of anhydrobiosis make it a fascinating area of study and, over the past 30 years, increasingly sophisticated biochemical, biophysical, physiological and molecular biological techniques have been employed to uncover its molecular mechanisms. However, the vast majority of such studies have been carried out with model, *in vitro*, systems and there is still much to learn about anhydrobiosis in the organisms themselves, as a review of what is known in the bdelloid rotifer will show.

3. ANHYDROBIOSIS IN ROTIFERS: A HISTORICAL SURVEY

(a) *Early studies*

Van Leeuwenhoek did not, in fact, think that the rotifers he observed were desiccated, instead proposing that, in their contracted form, their 'skins […] are of such a solid texture, that they do not permit the least evaporation: and, were it not so, […] these creatures in very dry weather, being deprived of water, must all perish' (Van Leeuwenhoek 1702, translated by Hoole 1807, (p. 213)). Other commentators and researchers following Van Leeuwenhoek, such as Joblot (1718), Baker (1742, 1753), Trembley (1747) and Hill (1752), were not specifically concerned with whether rotifers were dry or not, although clearly the idea that they and other anhydrobiotic animals might be in a resting state was pervasive at the time. Needham (1743) had recovered nematodes ('eel-worms') by rehydration of blighted wheat, but although Spallanzani (1765) initially strongly disputed their animal nature, such that Needham felt obliged to withdraw his original work (Keilin 1959), he was later vindicated by l'Abbé Roffredi (1775*a*,*b*, 1776) and Fontana (1776). Both workers showed independently that the 'fibres' present in the infected wheat grains were clearly alive, and went on to demonstrate the life cycle of the worms.

The discussion surrounding the work of Roffredi and Fontana, which took place in the early 1770s and was only published several years later, must have caused Spallanzani to revisit the phenomenon of anhydrobiosis. In 1776, Lazzaro Spallanzani (1729–1799), an Italian clergyman and distinguished professor of philosophy at the University of Modena, published his work *Opuscoli fisica animale et vegetabile* ('tracts on the natural history of animals and plants'), in which he argued against the church's position on spontaneous generation and provided evidence for the

preformation theory. This made Spallanzani not only one of the best known scientists of his time, but also one of the most controversial. Spallanzani devoted one of the chapters of the *Opuscoli* to the anhydrobiosis of rotifers. He established that, to survive, rotifers needed to be dried in sand, probably indicating a requirement for slow drying, and he made observations consistent with loss of body water: 'When dried, the solids are contracted and distorted, the whole body of the animal is reduced to a hard shapeless atom of matter; pierced by a needle, it flies in pieces like a grain of salt' (Spallanzani 1776, translated by Dalyell (1803), p. 137). Spallanzani furthermore showed that rotifers can be stored in the dried state for longer than 4 years and that warm water revives dried animals quicker than cold water. Dried rotifers were cooled or heated to temperatures the equivalent of at least –12 °C and 68 °C, respectively, and survived this treatment. These experiments caused Spallanzani to believe that all life processes stopped when the animals were dried. The overall quality and breadth of Spallanzani's work is astonishing, since by the late eighteenth century he had established the principles of anhydrobiosis, much as we understand them today. He was the first to propose clearly that rotifers did not retain their body water as their surroundings dried out, and that they became ametabolic in the dry state.

(b) *Dry or not dry?*

After Spallanzani, the matter rested for 62 years before Ehrenberg proclaimed, without the encumbrance of experiment, that rotifers are not truly desiccation tolerant, but that it is rather the eggs, hidden in the sand, which hatch after rehydration (Ehrenberg 1838). This belief was supported by other researchers at the time, including Bory St Vincent (Keilin 1959). The opposing view was taken by Doyère (1842) and Gavarret (1859), who published detailed experiments on rotifer anhydrobiosis. Doyère confirmed all of Spallanzani's experiments and established that slow drying was crucial for survival, as opposed to a specific requirement for sand. He stored dried rotifers in a vacuum of 5–6 mmHg, and the animals revived after rehydration. This led him to conclude that they are in a state of 'life in potentia'. Gavarret supported this view, after storing dried rotifers for 51 days in a vacuum of 4 mmHg and confirming survival. These findings were strongly disputed by Pouchet (1859*a–c*), however, who simply rejected the idea of 'life in potentia' and tried to prove spontaneous generation instead.

The discussion in the French literature of the time was so vigorous that a commission was appointed by the Société de Biologie in 1859 to resolve the matter. The report by the head of the commission, Paul Broca, was published in 1860 and contained many new experiments. The commission concludes that: 'The resistance of tardigrades and rotifers to elevated temperatures seems to increase further, once they have been previously desiccated. Rotifers can be revived after remaining in a dry vacuum for 82 days and being immediately afterwards heated to 100 °C for 30 min. Hence, animals […] brought to the most extreme point of desiccation that we can achieve in these conditions using current scientific knowledge, retain the ability to revive, once in contact with water' (Broca 1860, p. 139, translated by the authors). It is worth noting, however, that no direct measurements of residual moisture content have ever been made on dried rotifers. Strictly, therefore, the degree of desiccation in anhydrobiotic rotifers remains to be determined.

Broca's report should have decided the matter once and for all, but the dispute continued for several decades; many researchers remained unconvinced of the ability of some organisms to survive desiccation. This is perhaps not surprising at a time when researchers and philosophers were still arguing about spontaneous generation, prior to Pasteur's work in the early 1860s, and with the debate surrounding the Darwin–Wallace theory of natural selection underway after communications to the Linnean Society in 1858 and the publication of *The origin of species* in 1859. Thus, even towards the end of the nineteenth century, texts were published which denied anhydrobiosis (e.g. Zacharias 1886).

(c) *Phenomena associated with rotifer anhydrobiosis*

Overall, however, acceptance of the phenomenon became more widespread and the focus of attention shifted from whether, to how, rotifers survive desiccation. It is not an exaggeration to state that we are no nearer to answering this question now, a century and a half after Broca, and that most proposed mechanisms have ultimately proved disappointing. For example, in the 1870s, two authors suggested that rotifers secrete a gelatinous substance which protects them during desiccation (Cubitt 1872; Davis 1873). This led to some confusion among researchers, as no such substance had been reported by Broca (1860) and others before him. Although this idea was shown to be incorrect by Jacobs (1909), Bryce (1929) later claimed that two species of bdelloid rotifer, *Macrotrachela natans* and *P. de koningi*, produce a protective cyst in response to water loss. However, technical details are not clear in this publication and no further work on anhydrobiosis in these species has been published; encystment is not currently regarded as critical.

Janson (1893) observed that rotifers possess coloured carotenoid-containing particles in the stomach, which apparently disappear on drying. They were not present in starved rotifers, which did not survive desiccation, and Janson therefore assumed that these particles were vital for anhydrobiosis. Hickernell (1917) reported that, in *P. roseola*, food granules present in the stomach disappeared at the onset of desiccation and are absent in dried animals. Dobers (1915), however, claimed that: (i) no colour changes occur during desiccation; (ii) the coloured particles are storage compounds, which are used during starvation periods; and (iii) there are rotifers which do not produce storage compounds, yet are desiccation tolerant. Part of the confusion over these particles may be due to the fact that many bdelloid rotifer species feed on coloured algae which result in coloration of the gut. It is nowadays clear that this colour is evenly distributed throughout the stomach and is maintained during desiccation; food granules might be present in addition to this colour. Jacobs (1909) established that food availability has a strong impact on survival: 82% of well-fed *P. roseola* survived his drying protocol, whereas starved animals did not survive at all.

Jacobs (1909) also examined other factors which correlate with successful anhydrobiosis. He confirmed the

importance of slow drying, because rotifers dried rapidly in a desiccator exhibited poor survival (12%) compared with those dried slowly under a cover (75% survival). Temperature was also shown to have an effect, especially during storage of dried animals. Rotifers dried at 40 °C had a slightly higher survival rate (94%) than those dried at 20 °C (82%), but survival decreased during storage, although more slowly at 20 °C than at 40 °C. Dry storage conditions were also shown to be better than storage at high relative humidity.

Hickernell (1917) was the first to develop a fixation procedure for bdelloid rotifers, allowing in-depth cytological studies of rotifers in the dried and hydrated states. He found no difference in organ positioning between dried and control animals, but showed that the integument of the mid-trunk is structurally different from that of the head and the foot. It was suggested that this may be relevant to desiccation tolerance, because the animals pull in head and foot on dehydration, forming a structure sometimes referred to as a tun. Hickernell also claimed that chromatin associates with the nuclear membrane in dried animals, a result which was later confirmed by Dickson & Mercer (1967) for *P. roseola* by using the electron microscope. However, it is not known whether this phenomenon is restricted to anhydrobiotes, and, in general, no structural modifications have been linked to desiccation tolerance in rotifers.

(d) *Biostability*

Rahm published several articles on bdelloid rotifers, including work on the remarkable biostability conferred by anhydrobiosis (Rahm 1923, 1926, 1937). He showed that dried rotifers can be exposed to 151 °C for 35 min and still survive, and also reported that a bdelloid rotifer *M. quadricornifera* revived after having been stored in a dry moss sample for 59 years. Rahm is cautious, however, and stated that this may have been due to contamination with contemporary dust. He himself stored rotifers (in moss) at 0.1 mmHg pressure for 1 year and detected survivors. Rahm also claimed that dried animals (rotifers, nematodes and tardigrades) stored in a 100% nitrogen atmosphere do not survive the anhydrobiotic state at all, and therefore assumed that small amounts of oxygen were required for minutely slow metabolism. A later study was unable to detect signs of metabolism (Umezawa 1958), however, and Rahm's view is contrary to current opinion (Örstan 1998*a*). His results might be explained if the nitrogen was wet and the rotifers were therefore stored under unfavourable conditions. Rahm also reported on resistance to radiation in the dry state: exposure to strong ultraviolet light damaged dried animals, but they survived 'reasonably well', while hydrated animals were killed almost instantly. By contrast, X-ray exposure did not seem to visibly damage either hydrated or dehydrated animals. Rahm also cooled rotifers to very low temperatures (−190 °C) and found survivors. Later, Becquerel (1950) cooled dried rotifers of species *Habrotrocha constricta* and *P. roseola* to 0.05 K and demonstrated survival.

Günter Lindau extended the observations of Jacobs on storage conditions, showing that dried rotifers could be stored at −10 °C to −15 °C for 90 days without any drop in viability. Furthermore, he found that rotifers which were initially exposed to 85% relative humidity, and which were covered immediately after tun formation with a layer of gelatin and then dried to completeness, maintained viability. After 11–12 days of storage, probably at room temperature, they showed 95–100% survival (Lindau 1958).

(e) *Modern times*

Keilin (1959) reviewed anhydrobiosis extensively in his Leeuwenhoek lecture. This stimulated the work of Clegg, Crowe, Carpenter and other leading figures in the field, but rotifer anhydrobiosis was not widely studied in the 1960s and 1970s. In the past 20 years, it is largely the research of Claudia Ricci's group in Italy and that of Aydin Örstan in the USA which has added to our knowledge on rotifers. Ricci & Melone (1984) studied *M. quadricornifera* with a scanning electron microscope, but were unable to detect gross structural changes in dried animals which would indicate specific adaptations for desiccation tolerance. Ricci *et al.* (1987) reported that the internal clock of rotifers is arrested during anhydrobiosis: rotifers do not age during suspended animation in the sense that average lifespan depends on their age prior to desiccation, and not on the time spent in the dry state. Furthermore, for anhydrobiotic rotifer eggs, age was shown to affect survival: the older the eggs, the higher the survival rate, a result confirmed by Örstan (1995) for *Adineta vaga*. Schramm & Becker (1987) showed that bdelloids grown in aquaculture required a 'lag time' between desiccation cycles. Once a dried rotifer has been successfully rehydrated, the same animal cannot be subjected to a desiccation regime immediately, but requires a recovery period of at least 1 day. Örstan (1998*a*) performed a series of desiccation experiments on bdelloid rotifers in lichen samples. He disproved Rahm's hypothesis that dried rotifers cannot survive without oxygen because storage in an argon atmosphere proved better than storage in air. Furthermore, it was shown that, at room temperature, storage at intermediate humidities is better than very humid or dry conditions. As storage temperature is decreased, this effect becomes less important, until at temperatures below 0 °C, dried rotifers maintain viability over 18 months.

Ricci (1998) demonstrated that most bdelloid rotifer species can be considered anhydrobiotic, although some are clearly not, and found that the degree of survival of desiccation seems to be species dependent. Between 25% and 95% of individuals survive a given drying condition, with the best-surviving species being *P. rapida*. A later study showed that some species, like *M. quadricornifera*, survive rapid water removal, and others, like *P. roseola*, require a slower desiccation regime for maximum survival (Caprioli & Ricci 2001). This is reminiscent of observations on anhydrobiotic nematodes, which have been categorized as fast, or slow, dehydration strategists (Womersley & Higa 1998). Finally, Guidetti & Jönsson (2002) studied the long-term anhydrobiotic survival of rotifers. They investigated 63 individual moss and lichen samples from public and private collections, kept dry for between 9 and 138 years. Rotifers were identified in many of these and 13 survivors of the genus *Mniobia* were found in one 9-year-old sample, making this the longest well-documented period of rotifer anhydrobiosis.

An earlier review of rotifer anhydrobiosis is included in Gilbert (1974). For reviews covering the evolutionary and

ecological implications of rotifer anhydrobiosis, the reader is referred to Ricci (1987, 2001) and Örstan (1998*b*).

(f) *Summary*

What do 300 years of research into rotifer anhydrobiosis teach us? We know that successful entry into anhydrobiosis requires a slow, gentle desiccation regime. Once dry, storage conditions are crucial, with moisture, oxygen and temperature affecting survival. Anhydrobiosis confers a state of suspended animation in which life processes are halted—animals probably do not respire and their internal clock is arrested—and in which they can remain for many years. The dry state confers resistance to extreme environmental stresses, such as cooling to almost absolute zero, heating above the boiling point of water and exposure to ionizing radiation. Finally, there is a clear divide between species regarded as anhydrobiotic and those which are not. However, nothing is known about biochemical adaptations: how do rotifers actually achieve survival in the dry state and how does it relate to what is known in other anhydrobiotic organisms?

4. HOW DO ROTIFERS ACHIEVE ANHYDROBIOSIS?

Numerous reviews over the past decade, some of which are referenced here, have summarized research on anhydrobiosis and the various hypotheses for how it works (Crowe & Crowe 1992; Crowe *et al.* 1992, 1998, 2001; Potts 1994, 1999, 2001; Bartels *et al.* 1996; Ingram & Bartels 1996; Oliver 1996, 1997; Aguilera & Karel 1997; Sun & Leopold 1997; Oliver *et al.* 2000, 2001; Clegg 2001; Bartels & Salamini 2001; Hoekstra *et al.* 2001; Wright 2001). The continuous flow of such reviews reflects the fact that many questions remain to be answered about anhydrobiosis, even 300 years after Van Leeuwenhoek's observations, and that it is an increasingly active field.

(a) *Non-reducing disaccharides in anhydrobiosis*

Since the modern era of investigation into anhydrobiosis began in the late 1960s and the 1970s, non-reducing disaccharides have been linked with eukaryotic desiccation tolerance, specifically, trehalose (α-D-glucopyranosyl-(1-1)-α′-D-glucopyranoside) and sucrose (α-D-glucopyranosyl-(1-2)-β-D-fructofuranoside). Trehalose is found in anhydrobiotic animals, fungi and some resurrection plants (e.g. *Selaginella* spp.; Quillet & Soulet 1964; Gaff 1989), whereas predominantly sucrose, but also oligosaccharides such as raffinose and stachyose, is seen in resurrection plants such as *C. plantagineum* and in orthodox seeds (Oliver *et al.* 2000). While some bacteria can accumulate trehalose, for example, in response to increasing extracellular osmolarity (Strøm 1998), and anhydrobiotic cyanobacteria seem to contain small amounts of both sugars (Hershkovitz *et al.* 1991 and references therein; Hill *et al.* 1994), it is not clear to what extent they are important for prokaryote anhydrobiosis.

Trehalose was first associated with anhydrobiosis in animals in embryonic cysts of the brine shrimp *A. salina* (Clegg 1962, 1965, 1967). It was shown that in *Artemia* cysts trehalose is present at concentrations of *ca.* 15% of the total dry weight, corresponding to *ca.* 150 mM in the hydrated state. The biosynthesis of trehalose also correlates with acquisition of desiccation tolerance in nematodes (Madin & Crowe 1975; Womersley 1981; Perry 1999). In the tardigrade *Macrobiotus areolatus*, trehalose concentrations were found to increase in anhydrobiotic animals (Crowe 1975), although it accumulates to only *ca.* 2% of dry weight (*ca.* 20 mM) in *Adorybiotus coronifer* during dehydration (Westh & Ramløv 1991).

A few publications address the role of trehalose in yeast anhydrobiosis. As cultures of *S. cerevisiae* approach the stationary phase, they begin to accumulate trehalose, which correlates with an increased ability to withstand drying (Gadd *et al.* 1987). Trehalose levels increase further when stationary phase cells are dried (Payen 1949; Marino *et al.* 1989). During the log phase of growth, yeast cells contain little trehalose and correspondingly show poor survival of desiccation. However, log-phase cells can be induced to synthesize trehalose by heat stress, when they become more resistant to drying (Hottiger *et al.* 1987). Further experiments indicate that extracellular trehalose is also important because mutants in a trehalose transporter gene, whose protein normally exports the disaccharide to the extracellular milieu, have markedly reduced desiccation tolerance (Eleutherio *et al.* 1993).

In plant seeds, production of high concentrations of sucrose coincides with maturation and the acquisition of desiccation tolerance (Oliver *et al.* 2000). In the resurrection plant *C. plantagineum*, the novel sugar 2-octulose is replaced by sucrose as leaf tissues dry, such that sucrose represents 90% of total sugar content (Bianchi *et al.* 1991) and, accordingly, sucrose synthase genes are upregulated by dehydration (Kleines *et al.* 1999). It therefore seems that intracellular accumulation of disaccharides is linked to anhydrobiosis in a wide range of organisms (Crowe *et al.* 1992; Aguilera & Karel 1997). However, perhaps surprisingly, there has been no investigation of the sugar content of rotifers undergoing anhydrobiosis. We investigate this below.

(b) *Multiple roles of disaccharides in the anhydrobiotic cell*

Why are trehalose and sucrose thought to be important for anhydrobiosis? We can address this by considering some of the events occurring during preparation for, and entry into, anhydrobiosis. For a vegetative anhydrobiotic cell, three overlapping stages can be identified during which different stresses operate.

In the first, dehydration, stage, bulk water is lost progressively throughout the cell, although macromolecules and phospholipids initially remain fully hydrated. This leads to the concentration of intracellular solutes, changes in pH and increased viscosity, all of which can destabilize native protein conformations by disturbing hydrogen bonds, Van der Waals forces and hydrophobic interactions (Stryer 1999). The cell eventually enters a state where metabolism is restricted and biochemical networks begin to break down (Clegg 1978; Womersley 1981). During this period, when the cell is experiencing stress but is still metabolically active and can mount a stress response, any adaptations required for survival of desiccation should appear, if they are not present constitutively. In anhydrobiotic nematodes and tardigrades, trehalose is accumulated at this time; in maturing plant seeds and the tissues of resurrection plants, sucrose becomes abundant; and it

is the solution properties of these molecules which are thought to be important during the dehydration stage.

Trehalose is one of the so-called compatible solutes, which are used by plants, animals and micro-organisms to counteract osmotic stress (Yancey *et al.* 1982; Galinski *et al.* 1997; Strøm 1998). Because it is non-toxic and relatively highly soluble, it can be accumulated in large amounts inside the cell to help counterbalance elevated extracellular osmolarity. Compatible solutes may also be important during the early stages of drying, where a single-celled organism is drying from an aqueous environment, or where tissue fluids are becoming increasingly concentrated in a multicellular organism. However, the effect of the sugar is not purely osmotic: trehalose (and sucrose would behave similarly, although it is less commonly used as a compatible solute) is partially excluded from the hydration shell of biopolymers, a situation which increases order in the system. The degree of this entropy decrease is proportional to the surface area of proteins and other biopolymers within the cell, but is minimized when these macromolecules retain their native conformation, because a larger surface area is exposed when they unfold. This is the principle of preferential exclusion (Arakawa & Timasheff 1982; Timasheff 1992) and it is thought to be the mechanism whereby disaccharides and other compatible solutes protect biopolymers against denaturing stresses caused by elevated osmolarity and, similarly, by dehydration. This idea has been developed further by Bolen and colleagues, who have shown that the preferential exclusion is due to an unfavourable interaction between the compatible solute and the peptide backbone of proteins, the so-called osmophobic effect (Bolen & Baskakov 2001). In this respect, disaccharides act as thermodynamic stabilizers, because structural changes become more energetically unfavourable in their presence.

The second, desiccation, stage is reached when water content in the cell dips below 0.3 g g dry weight^{-1}, and particularly when it reaches 0.1 g g dry weight^{-1}, where metabolism effectively ceases. This is what Clegg calls the 'ametabolic domain', where water is in such short supply that macromolecules are not fully hydrated, which would normally lead to structural instability. In anhydrobiotes, it is proposed that disaccharides behave as water replacement molecules, thereby preventing irreparable structural damage to biopolymers and membranes as they dry and enter the solid phase. The water replacement hypothesis, developed by Clegg (Clegg 1974, 1986; Clegg *et al.* 1982) and Crowe (Crowe & Madin 1974; Crowe *et al.* 1992) from earlier discussions (Clegg 1967; Crowe 1971; Crowe & Clegg 1973), and with reference to Warner (1962) and Webb (1965), indicates that these polyols form hydrogen bonds, as does water, with the head groups of membrane phospholipids, and with the surfaces of nucleic acids and proteins. In this way, the sugars are thought to take water's part in maintaining the structure of macromolecules and membranes. A number of the reviews quoted earlier have summarized this hypothesis.

It is worth commenting that, moving from the dehydration stage, where disaccharides are partially excluded from biopolymer surfaces, to the desiccation stage, where sugars are intimately associated by hydrogen bonding with such surfaces, defines a transition state within the anhydrobiotic cell. Clegg *et al.* (1982) reported nuclear magnetic resonance (NMR) data which are interpreted as providing evidence for such a transition: as *Artemia* cysts are progressively dried, the self-diffusion coefficient (D) and relaxation times (T_1 and T_2) for water protons decrease accordingly. However, when the ametabolic domain is entered, below 0.3 g H_2O g dry weight^{-1}, minima are reached in all three curves, and values suddenly increase again to a threshold. It was argued that this demonstrates release of residual water from macromolecule hydration shells, allowing increased rotation and translation. Thermal analyses and NMR experiments in dried yeast suggested that trehalose levels of 2–3% of dry weight (20–30 mM) are sufficient to effect this water replacement, although survival of desiccation continued to improve at higher trehalose levels (Sano *et al.* 1999). Other data in support of the water replacement hypothesis come largely from *in vitro* work with protein and membrane systems dried with or without sugars (Crowe *et al.* 1998). However, there is little evidence, particularly in whole cells or organisms, which demonstrates the transition between the thermodynamic effect of protective disaccharides in solution, and their role as water replacement molecules in the solid, dry phase.

For the third, dry, stage, representing anhydrobiosis proper, which can potentially extend for long periods of time, there is increasing information on the nature of the solid phase, where the cytoplasm is thought to form an amorphous organic glass during desiccation (Burke 1986). This drastically alters the reaction kinetics of the system, so that, even though they might be thermodynamically favourable, any structural changes in biopolymers and membranes occur at a rate which is slow in comparison to the duration of the stress. It is proposed that disaccharides are instrumental in the vitrification process, owing to their high intracellular concentrations; sugars like trehalose and sucrose act as kinetic stabilizers, increasing cytoplasmic viscosity, ultimately leading to vitrification of the cytoplasm: the so-called vitrification hypothesis (Sun & Leopold 1997; Crowe *et al.* 1998).

Molecules embedded in a sugar glass would be trapped in space and time, as molecular diffusion, and the chemical reactions requiring it, would effectively come to a halt. Critical to the hypothesis is that the glass transition temperature—T_g, the temperature which marks the inflection point between the glassy state and the looser, although still highly viscous, 'rubbery' state—should be high relative to ambient temperatures likely to be experienced by the organism. T_g is dependent on water content of the glass, increasing as water is removed (Franks 1985; Sun & Leopold 1997): the drier the glass, the more (thermo)stable it is, although some degradative reactions continue, at a slower rate, below T_g (as reviewed by Crowe *et al.* 1998). The glassy state has been observed in various plant seeds, *Artemia* cysts and commercial preparations of bakers' yeast (Sun & Leopold 1997; Crowe *et al.* 1998; Cerrutti *et al.* 2000; Schebor *et al.* 2000), although clearly glass stability depends on storage temperature and moisture content of the anhydrobiote. It has been proposed that vitrification is important for the long-term stability of anhydrobiotes in the dry state (Sun & Leopold 1997). However, it has been cogently argued (Crowe *et al.* 1998) that vitrification itself is not enough—for example, excellent glass formers such as dextran are not effective at stabi-

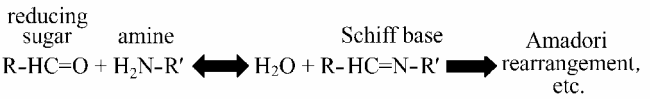

Figure 3. The Maillard reaction. Formation of a Schiff base between the carbonyl group of a reducing sugar and the amine group of a protein or nucleic acid. The molecule of water evolved is removed under conditions of low water activity, shifting the equilibrium to the right.

lizing biomolecules *in vitro*—and that native biomolecule conformation must be maintained during the dehydration and desiccation stages.

Stresses which operate in the dry state include the Maillard and Fenton reactions, oxidation and irradiation. Such damaging chemistry will be reduced in the glassy state, but trehalose and sucrose may also have some specific protective activities. For example, the Maillard reaction (Maillard 1912)—also referred to as non-enzymatic browning—takes place between the carbonyl group of reducing sugars and the amine groups of proteins and nucleic acids, leading through a complex cascade of rearrangements to polymeric, pigmented end products. The initial formation of the Schiff base is an equilibrium reaction, which evolves a molecule of water (figure 3). Under conditions of high water activity, the equilibrium is driven strongly to the left of the equation shown, but in the dry state there is strong pressure in the forward direction owing to rapid removal of water. Maillard activity is a major source of damage in stored, dry biologicals (Lea *et al.* 1950), but both trehalose and sucrose are non-reducing disaccharides, and therefore should not participate. The glycosidic bond in sucrose is relatively weak, however, and when hydrolysed gives glucose and fructose, which readily form Maillard products with amine groups at low water activity (O'Brien 1996). Trehalose is rather more stable and is less likely to undergo the browning reaction (El-Nockrashy & Frampton 1967); indeed, trehalose inhibits its progression (Loomis *et al.* 1979), and this is probably significant for anhydrobiosis, as originally noted by Crowe & Clegg (1973).

Oxidative damage, through the generation of free radicals, is also thought to be incurred during desiccation and the period in the dry state (Heckley 1978; Senrartna & McKersie 1986). Antioxidants which neutralize free radicals, including enzymes such as superoxide dismutase and catalase, are induced during dehydration in plants (Smirnoff (1993); Farrant (2000) and references therein) and animals (Browne 2001), but their activity will be compromised as water deficit becomes extreme. However, sugars and sugar alcohols such as galactose, *myo*-inositol, sorbitol and mannitol are known to scavenge free radicals (Smirnoff & Cumbes (1989) and references therein). In principle, both trehalose and sucrose, the main sugars associated with anhydrobiosis, could also have this property and, indeed, trehalose has been indicated in oxidative stress protection of yeast (Benaroudj *et al.* 2001).

In summary, trehalose and sucrose are a recurring theme in anhydrobiosis, at least among eukaryotes, and may have multiple protective roles.

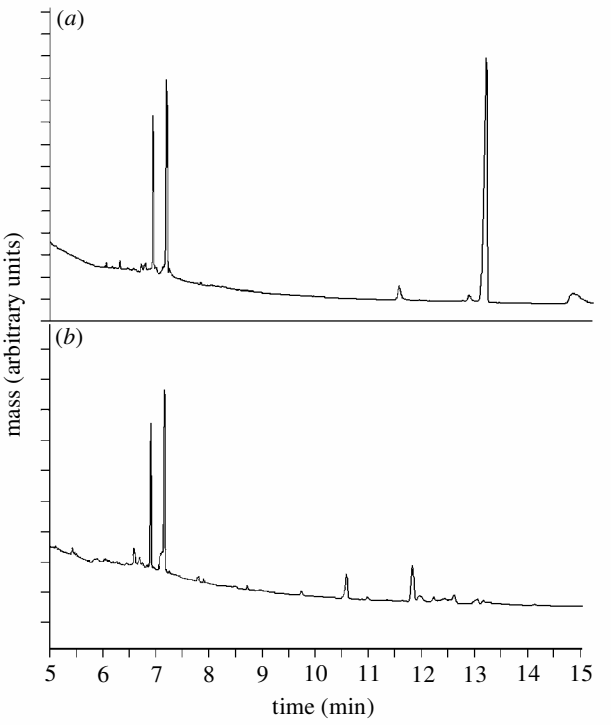

Figure 4. Carbohydrate analysis by gas chromatography. (*a*) Carbohydrates extracted from dried nematode *Aphelenchus avenae*, containing glucose and accumulated trehalose. (*b*) Carbohydrates extracted from dried rotifer *Philodina roseola*, containing glucose, but no trehalose or other disaccharide. Glucose is represented by the doublet of peaks emerging at 7 min, while trehalose gives a single peak at 13 min.

(c) *Anhydrobiosis without trehalose in bdelloid rotifers*

The analysis of sugar accumulation in the rotifers *P. roseola* and *Adi. vaga* as a response to desiccation stress gave surprising results. Both rotifer species were collected from a birdbath in Cambridge, UK, and clone cultures established from single individuals; these can be expanded to hundreds of thousands or millions of animals as required. The rotifers were subjected to a drying regime in which animals were placed between two wet filter papers and incubated in a sealed dish for 2–3 days. The cover was then removed and the animals were allowed to air dry for another 3 days. This resulted in excellent recovery after rehydration of between 75% and 80% of the animals, in line with Ricci's (1998) results for these species. By contrast, rotifers dried immediately over silica gel showed a markedly lower survival of *ca.* 12%, as expected from the literature. Analysis of the carbohydrate content of dried rotifers was performed by gas chromatography (GC). The nematode *Ap. avenae*, dried slowly according to established protocols which elicit extensive accumulation of trehalose (Madin & Crowe 1975; Browne 2001), was used as a reference. The GC data showed the expected large increase in trehalose content on drying *Ap. avenae*, but no such change in either rotifer species; indeed no trehalose was observed at all (figure 4). Furthermore, it was apparent that no other simple sugar was produced in detectable quantities during dehydration, as mono-, di- and small oligosaccharides would be detected on the chromatograph. Sucrose, when added to the samples as a standard, and intracellular glucose are clearly detected. Glucose

constitutes *ca*. 1% of dry weight in the nematode *Ap. avenae* (Browne 2001) and similar quantities were obtained for the rotifers *P. roseola* and *Adi. vaga*; trehalose comprises 10% of dry weight in desiccated nematodes. It is therefore reasonable to assume that we would detect changes in the disaccharide profile at least in this range (i.e. 1–10% of dry weight), which overlaps with trehalose concentrations expected in anhydrobiotic animals.

Trehalose was also not detected in rotifers subjected to other stresses which might be expected to provoke its biosynthesis (i.e. heat, salt, cold). If rotifers are able to produce trehalose, they are likely to use the two-step pathway found in all other eukaryotes whose first step is the transfer of glucose from UDP-glucose to glucose-6-phosphate to yield trehalose-6-phosphate. This step is catalysed by trehalose-6-phosphate synthase (EC 2.4.1.15), an enzyme whose sequence is highly conserved from Gram-negative bacteria to nematodes and higher plants (Blázquez *et al.* 1998; Vogel *et al.* 1998). A polymerase chain reaction (PCR) method with degenerate oligonucleotide primers based on conserved protein sequence can be devised to clone the respective gene (*tps*) from a wide range of species. However, although *tps* genes have been isolated using this technique from species including nematodes, fruitflies, yeast and *E. coli*, we have been unable to amplify PCR fragments from rotifer genomic DNA, indicating that *tps* genes might not be present at all in *P. roseola* and *Adi. vaga*.

Finally, we examined the metabolite profile of dried *Ap. avenae* and *P. roseola* using proton NMR spectroscopy. In agreement with the GC experiments, NMR analysis of extracts of *Ap. avenae* showed that the general metabolite content is raised in the dried, compared with the hydrated, state; peaks corresponding to trehalose are markedly increased in area. However, no such changes could be detected in the rotifers, suggesting that the overall representation of metabolites is not significantly altered in preparation for anhydrobiosis (Lapinski *et al.* 2003).

5. REAPPRAISAL OF THE ROLE OF DISACCHARIDES

The lack of trehalose in rotifers is profoundly disturbing for our understanding of anhydrobiosis: as outlined above, non-reducing disaccharides are the cornerstones on which the various explanatory hypotheses are based. Not only is no trehalose present, however, but there is no apparent change in the small-molecule profile in dehydrating animals. Therefore, no analogue of trehalose is produced during entry into anhydrobiosis, despite the requirement for preconditioning by slow drying, which is normally associated with adaptive changes. So what is going on here?

(a) *Re-examination of the link between disaccharides and anhydrobiosis*

Perhaps we need to look again at the association of trehalose or sucrose with anhydrobiosis: how secure is it? Certainly in prokaryotes the link is tenuous. Desiccation-tolerant *Lactobacillus plantarum* apparently contains only insignificant quantities of endogenous sugars (Linders *et al.* 1997). In desiccating cells of the cyanobacterium *Nostoc commune*, trehalose represents about 1 mg g dry weight^{-1}, and sucrose only 0.8 mg g dry weight^{-1}, i.e. 0.1% and 0.08% of dry weight, respectively (Hill *et al.* 1994). Although it cannot be ruled out that surviving cells might contain higher concentrations of sugars at the expense of non-survivors, an equal distribution throughout the population would amount to no more than 1 mM intracellular trehalose prior to desiccation and is probably not sufficient to stabilize cytoplasmic components during anhydrobiosis. The *D. radiodurans* genome contains genes for trehalose synthesis (White *et al.* 1999) and *Deinococcus* spp. are known to produce and excrete this sugar in small amounts (Kizawa *et al.* 1995); but its significance in desiccation tolerance has not been determined, although this is currently being examined (J. Battista, personal communication).

What about tardigrades and nematodes? There is a single report showing that tardigrades accumulate trehalose up to 2% of dry weight (*ca*. 20 mM) in response to desiccation (Westh & Ramløv 1991). This is, however, the approximate amount of trehalose in the nematode *Ap. avenae* before it starts to respond to desiccation stress, and before acquisition of desiccation tolerance (Madin & Crowe 1975; Higa & Womersley 1993; Browne 2001). Trehalose concentrations in tardigrades seem rather lower than might be expected, and its importance is therefore unclear (Wright 2001), although interestingly Sano *et al.* (1999) proposed that this is approximately the level at which water replacement is complete. Nevertheless, even in anhydrobiotic nematodes, where trehalose is produced at higher concentrations, the correlation between trehalose accumulation and acquisition of desiccation tolerance is somewhat loose (Womersley & Higa 1998). Higa & Womersley (1993) noted that, during dehydration, trehalose concentrations in *Ap. avenae* reached maximal levels before full desiccation tolerance was attained. Browne (2001) has made similar observations on the later stages of the drying protocol, but also noted that in some dehydration protocols, trehalose content does not increase markedly during the first 12 h of desiccation stress, despite survival levels increasing from 0% to 6% (figure 5*a*).

A similar situation pertains in yeast. Gadd *et al.* (1987) determined that *S. cerevisiae* produces very high concentrations of intracellular trehalose on entry into stationary phase, increasing from basal levels of *ca*. 35 mM to *ca*. 400 mM, and that this correlates with improved survival of desiccation, from 0% to 10%. Close inspection of the data points engenders some caution, however. First, trehalose concentration increased rapidly to 200 mM within 4 days in the stationary phase. At the same time, survival increased to *ca*. 1%. Maximum trehalose concentration is reached after 8–10 days and approaches 400 mM. At this point, survival has still only increased to *ca*. 4% and it is not until 16 days in the stationary phase that maximum desiccation tolerance can be measured (figure 5*b*).

The conventional explanation for these observations in nematodes and yeast is that elevated intracellular trehalose levels are necessary, but not sufficient, for entry into anhydrobiosis, and that other adaptations are occurring during the dehydration stage. In yeast, where desiccation experiments are typically performed with stationary-phase cells, nutrient and oxygen limitation would stimulate the general stress response: many genes governed by STRE (stress response element) promoter elements

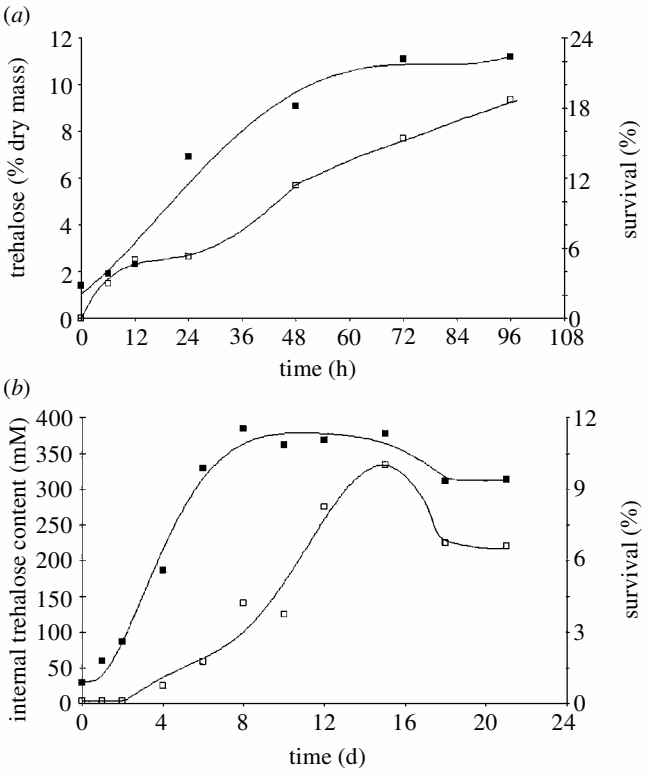

Figure 5. Trehalose content and survival of desiccation of anhydrobiotic organisms. (*a*) *Aphelenchus avenae*: trehalose accumulation (filled squares) and survival (open squares) after different periods of preconditioning at 90% relative humidity and subsequent drying over silica gel (redrawn from data of Browne 2001). (*b*) *Saccharomyces cerevisiae*: trehalose accumulation (filled squares) and survival (open squares) during growth and subsequent drying. Stationary phase is reached after 3–4 days in this slow-growth-rate protocol (redrawn from data of Gadd *et al.* 1987).

would become active (Ruis & Schuller 1995), including *TPS1*, which encodes trehalose-6-phosphate synthase (Winderickx *et al.* 1996). Similarly, in heat-shocked yeast cells, whose desiccation tolerance is increased (Hottiger *et al.* 1987), a number of genes controlled by heat shock element (HSE) promoter sequences would be transcribed (Estruch 2000). Some genes, including heat shock protein gene *HSP104* (Grably *et al.* 2002), and presumably *TPS1*, can be driven by both STRE and HSE sequences.

If trehalose is, however, necessary in these organisms, mutation of trehalose synthase genes should lead to a drastic reduction in anhydrobiotic capacity. Surprisingly, no experiments of this kind have been carried out, perhaps owing to technical difficulties. The study performed by Coutinho *et al.* (1988) used strain variants of *S. cerevisiae* with reduced capacity to produce trehalose. Strikingly, desiccation tolerance of wild-type and low-trehalose-level variants was very similar, although another strain overproducing trehalose seemed to show increased survival. This could suggest that intracellular trehalose is of limited importance in yeast desiccation tolerance. However, the strain variants used are uncharacterized, and it would therefore be of great interest to repeat these experiments with defined *TPS1* mutants.

Panek's group also looked at the effect of extracellular trehalose on survival. Wild-type yeast strains were compared with strains with a non-functional trehalose carrier, which is apparently responsible for transporting trehalose across the membrane into and out of the cell, but with unimpaired ability to produce trehalose (Eleutherio *et al.* 1993). Strains with the non-functional trehalose transporter were found not to survive the desiccation protocol used—despite producing up to 3.5% of dry weight (*ca.* 35 mM; in the wild-type range) trehalose intracellularly—whereas *ca.* 40% survival was observed in the wild-type control. When a 10% w/v (i.e. *ca.* 300 mM) trehalose solution was added as a drying excipient, survival of the variant rose to more than 40%. These results are also consistent with intracellular trehalose playing a minor role in yeast desiccation tolerance, and point to a more critical role extracellularly. Work with Gram-negative bacteria also indicates that extracellular addition of excipients is more important than elevated intracellular trehalose levels (García de Castro *et al.* 2000*a*; Manzanera *et al.* 2002).

The eukaryotic structure with the most impressive anhydrobiotic credentials is the orthodox plant seed. Seeds are well known to produce very high concentrations of sucrose in particular, and its accumulation coincides with increased desiccation tolerance (e.g. Oliver *et al.* 2000). In developing soybeans, for example, conditioning of seeds at high relative humidity increases sucrose, raffinose and stachyose levels. Almost 100% of preconditioned seeds survive desiccation, whereas unconditioned seeds are not viable (Blackman *et al.* 1992). Nevertheless, the accumulation of sucrose is not sufficient for anhydrobiosis, as shown by abscisic acid (ABA)-insensitive mutants in the *abi3* locus (*abi3-3* and *abi3-4*) of *Arabidopsis thaliana*, whose seeds lack desiccation tolerance, although the accumulation of sucrose is unhindered and is actually higher than in wild-type (Ooms *et al.* 1993). Interestingly, raffinose and stachyose were not present at all in the mutants, compared with 2% and 5% of dry weight, respectively, in the wild-type, which at first sight might point towards a role of those sugars in anhydrobiosis. However, the recalcitrant, i.e. desiccation-sensitive, seeds of *Avicennia marina* (grey mangrove), for example, contain high levels of carbohydrates, including stachyose, and yet do not survive drying (Bewley & Black 1994). Conversely, certain plant seeds do not contain significant amounts of sugars and yet are still desiccation tolerant. One example is the seeds of *Ricinus communis* (castor bean), which contain only negligible amounts of sugars (Bewley & Black 1994), but desiccate to residual moisture contents below 3.75% with a 100% survival rate on germination. This desiccation tolerance is only acquired at days 20–25 after pollination, suggesting that specific adaptations take place during maturation of castor bean seeds which confer anhydrobiotic capabilities (Kermode & Bewley 1985).

Further contributions to the debate on the role of disaccharides come from attempts to create anhydrobiotic cell types from desiccation-sensitive cells, an approach we have called 'anhydrobiotic engineering' (García de Castro *et al.* 2000*b*). Current excitement surrounds work on mammalian cells, which are normally sensitive to dehydration: loss of up to 65% of cellular water from cell lines (e.g. mouse L cells) can be tolerated, but further loss is fatal (e.g. Mansell & Clegg 1983). Therefore, cell engineering to improve desiccation tolerance will be necessary to achieve a viable dry state, and the cytostability it con-

fers. If successful, this would enhance our understanding of anhydrobiosis, as presumably a minimum set of adaptations would be defined for a particular cell type. But, additionally, mammalian cells would become 'off-the-shelf' items, stored in the dry state instead of deep frozen, which would greatly reduce storage costs for cell banks or for products based on cell technology, such as monoclonal antibody-producing hybridoma cell libraries or some types of biosensor. This approach may also be applicable to tissue engineering, which relies on cell-based therapies including the use of cells for tissue repair and as drug delivery modules, and to cell factories producing bioactive molecules. The prize for success is therefore considerable.

To date, anhydrobiotic engineering of mammalian cells has been attempted largely by the generation of intracellular trehalose, because it is not naturally produced by mammals. A question central to such projects is how high the intracellular concentration of trehalose should be for a reasonable expectation of success. Recognized eukaryotic anhydrobiotes define the physiological range as from *ca.* 20 mM (in tardigrades) to *ca.* 400 mM (in yeast). Mouse cell lines containing bacterial trehalose synthase genes can be induced to accumulate up to 100 mM trehalose, but although these cells show increased tolerance of high osmolarity, they do not survive desiccation, achieved by a number of different methods, even in the presence of extracellular trehalose (García de Castro & Tunnacliffe 2000; Tunnacliffe *et al.* 2001). Similarly, when an inducible pore is used to load mouse cells with concentrations of up to 400 mM trehalose, viability is not maintained after drying, although membrane integrity is preserved with concentrations of 200 mM or above if cells are stored at sub-zero temperatures (Chen *et al.* 2001). These results indicate that intracellular trehalose concentrations within the physiological range are insufficient to confer desiccation tolerance, and are consistent with the current understanding of anhydrobiosis. By contrast, Wolkers *et al.* (2001) have reported freeze-drying of human platelets loaded with only *ca.* 20 mM trehalose, with retention of function. Although this concentration is 5–10-fold lower than that reported by Chen *et al.* (2001) for maintenance of membrane integrity, it is possible that this is sufficient for non-replicating cell fragments. This argument does not explain the results of Guo *et al.* (2000), however, who claim to have improved desiccation tolerance in human cells containing no more than 1 mM trehalose, well below the minimum concentration found in anhydrobiotic animals. More remarkably still, the same group has reported similar success without any biochemical modifications to the cell (Gordon *et al.* 2001; Puhlev *et al.* 2001). However, there are technical questions relating to this work, particularly regarding the degree of cell desiccation, which need to be resolved (García de Castro *et al.* 2000*b*).

It is our opinion that trehalose alone is unlikely to deliver true desiccation tolerance for anhydrobiotic engineers, and, indeed, researchers are beginning to investigate other possibilities. A preliminary report from Bloom *et al.* (2001) outlines the use of hydrophilic extracellular glycan from the cyanobacterium *N. commune* to overlay mammalian cells prior to drying. The authors describe some recovery of viable cells, but an important question with this approach, as with that of Guo and colleagues, is whether treated cells have the low moisture contents typical of dried anhydrobiotes. Other adaptations apparently related to anhydrobiosis, including the scavenging of reactive oxygen species, downregulation of metabolism, and the accumulation of amphiphilic solutes, hydrophilic proteins or peptides, heat shock proteins or chaperones, may also provide protection during dehydration (Oliver *et al.* 2001), but have yet to be assessed for anhydrobiotic engineering. If these possibilities need to be tested in combination, however, it could be many years before fully desiccation-tolerant mammalian cells are produced.

6. CONCLUSIONS

What does all of this mean for disaccharides as cell-preserving agents? *In vitro*, there is very strong evidence for stabilizing properties in the dry state: enzymes, antibodies, nucleic acids, some viruses, liposomes and other membrane systems can be preserved using non-reducing sugars as drying excipients (see reviews cited above). However, *in vivo* experiments on recognized anhydrobiotes and attempts at anhydrobiotic engineering are less conclusive.

It is apparent that the presence of high concentrations of trehalose or sucrose is not sufficient for anhydrobiosis. Evidence in support of this conclusion includes the presence of disaccharides in recalcitrant seeds, the results of attempts at anhydrobiotic engineering of mammalian cells and the high disaccharide concentrations in yeast and nematodes which are not fully desiccation tolerant. Moreover, there is little to suggest that trehalose or sucrose is actually necessary for successful anhydrobiosis: the literature is sparse and unconvincing; we have been unable to detect trehalose or any other disaccharide in two bdelloid rotifer species; and there are many bacteria and some plant seeds which survive desiccation without significant disaccharide accumulation. We should stress that this does not mean that disaccharides are not critical for anhydrobiosis in some or, indeed, many species, but point out that the data to demonstrate this conclusively in living systems are lacking. Because it is apparent that, in some cases at least, anhydrobiosis is possible without disaccharides, some key *in vivo* experiments are needed to test the requirement for sugars in anhydrobiosis, the most important of which will employ mutants whose disaccharide synthase genes have been inactivated. Some of these mutants, in yeast, for example, have already been produced for other purposes and should help establish whether disaccharides confer anhydrobiosis in those organisms.

If there are questions about the role of disaccharides in anhydrobiosis, what else has been learnt, in three centuries of research, about which we can be more certain? Arguably, there is not a great deal; but two things have been firmly established. First, the intrinsic environment is important, with many organisms requiring some form of stress, and time to respond to that stress, to prepare for anhydrobiosis. This need not necessarily be desiccation stress; in yeast, it could be nutrient depletion and other stresses associated with the stationary phase, or heat stress (Hottiger *et al.* 1987), or osmotic stress (Eleutherio *et al.* 1997); in *C. plantagineum* callus, it could be ABA treatment (Ingram & Bartels 1996). Most stress responses have a number of adaptations in common, i.e. the general stress

response; in *E. coli*, this involves 50–100 genes (Hengge-Aronis 2002). In yeast, and perhaps nematodes and tardigrades, this general stress response may involve the accumulation of trehalose to varying degrees (Singer & Lindquist 1998; Argüelles 2000). In *Arabidopsis*, inactivation of genes which are part of stress response pathways can lead to loss of desiccation tolerance in seeds (Ooms *et al*. 1993). Although no disaccharide accumulation has been found to occur in rotifers, a slow desiccation regime in combination with well-fed animals is stringently required for high-level survival of desiccation, indicating that the animals mount some form of energy-expensive stress response. Second, extrinsic factors are also crucial to successful anhydrobiosis. Slow, gentle water removal, elevated temperatures during drying, dry storage conditions, low storage temperature, humidification before rehydration and elevated water temperature during rehydration can influence anhydrobiosis positively. Survival can be greatly improved or greatly reduced, depending on the conditions chosen. However, to our knowledge, no desiccation protocol has been devised which enables non-anhydrobiotes to undergo anhydrobiosis successfully.

These reflections point towards a qualitative, rather than a quantitative, difference between organisms regarded as anhydrobiotic, and organisms which are not so. Bdelloid rotifers are good examples: they are either desiccation tolerant or they are not (Örstan 1998*a*; Ricci 1998), which means there must be something special about those which are anhydrobiotic. If we are to hope to understand anhydrobiosis, we must continue to search for what is special about them, and draw on the full power of current and emerging techniques in pursuit of an answer. In organisms where a complete genome sequence is available, e.g. *S. cerevisiae* and *Ara. thaliana*, microarrays of all recognized genes can be used to determine which transcripts are induced by desiccation stress. With appropriate experimental design, this should lead to the identification of those genes which are specific to this stress response. Complementary information will be derived from experiments in proteomics and metabolomics. Where genome sequence is unavailable, direct analysis of differentially expressed mRNAs and proteins can be attempted. It is envisaged that a key panel of genes and proteins—the anhydrobiotic gene set, and corresponding anhydrobiotic protein set—are induced in rotifers in response to water stress, and that these directly govern anhydrobiosis, above and beyond any general stress response. A similar approach in resurrection plants, where Bartels's group is perhaps furthest advanced, has identified many induced genes whose function is under investigation (Ingram & Bartels 1996; Bartels & Salamini 2001). This strategy should prove especially valuable in rotifers, where attention need not be distracted by disaccharide accumulation.

7. VAN LEEUWENHOEK'S LEGACY

Antony van Leeuwenhoek (1632–1723) was born in the Dutch town of Delft, where he was educated at a grammar school until the age of 16. After the death of his father, Van Leeuwenhoek became an apprentice linen draper and subsequently established himself as a textile merchant, where he must have used magnifying glasses routinely to examine the quality of the cloth. He crafted his own single-lens instruments which at the time outperformed, in magnification and resolution, all existing compound microscopes. He was inspired by Hooke's *Micrographia* to look for subjects other than cloth, as shown by an early study of a bee sting, which he reported to The Royal Society in 1673. He communicated his research in more than 300 letters, of which 190 were sent to The Royal Society, and is considered the founder of microbiology through his observations of bacteria and other microscopic organisms.

Anhydrobiosis was just one of Van Leeuwenhoek's many discoveries, but it is perhaps one of the most enduring puzzles he has bequeathed to us. His sense of wonder at the resurrection performed routinely by a simple aquatic invertebrate, the bdelloid rotifer, is just as relevant today as it was then. The rotifer still has much to teach us, even 300 years after Van Leeuwenhoek, about an intriguing mystery of nature.

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